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Laboratory Protocol for Induction of Sporulation in *Phytophthora cajani* causing Phytophthora Blight in Pigeonpea

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ABSTRACT

Background: Phytophthora blight (PB), caused by *Phytophthora cajani* is a prominent disease in the low laying areas combined with intermittent rainfall. Induction of zoospores and sporangia of *P. cajani* in culture plate is troublesome and also limited information is available on the protocol for sporulation of sporangia and zoospores in the laboratory, The study developed and validated the protocol for induction of sporangia and zoospore of *P. cajani*.

Methods: The Protocol using 5-7 days old culture, diluted tomato extract broth and other culture conditions like the temperature of 30°C, alternate 12 hours of fluorescent light (2000 Lx) and dark can induce abundant sporangia and zoospores *in vitro*.

Result: The study developed and validated the new protocol for uniform and profuse induction of sporangia and zoospores of *P. cajani*. Diplanetism mechanism of *Phytophthora* spp. was recorded with *P. cajani* the findings are the first of its kind. Additionally, the study revealed the concentration of $1x10^{-5}$ zoospores/ml is optimum to develop the infection in plants with the shortest incubation period of 24 hours.

Key words: Diplanetism, *Phytophthora*, Pigeon pea blight, Sporangia, Sporulation, Zoospores.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important food grain legume crop cultivated worldwide under rainfed and semiarid conditions. India alone contributes 72% of the area and 66% portion of the global pigeonpea production with an area of 5.41 m ha with an annual production of 4.49 million tons (FAO, 2016). Pigeonpea is very important pulse crop for vegetarian population of the world as it supply the good amount of dietary protein and essential amino acids like methionine, lycine and tryptophan. It is a multipurpose crop, being grown not only for grain legume but also for fuel and fodder (Nene and Sheila, 1990). It is grown under a wide range of cropping systems on the Deccan Plateau (DP) in India (Reddy *et al*., 1998).

Phytophthora blight caused by *Phytophthora cajani* is third most important disease after Fusarium wilt and sterility mosaic disease (Kannaiyan *et al*. 1984). The first suspected occurrence of Phytophthora blight on Pigeonpea in India was reported in 1966 by Williams *et al*. (1968). Though the disease is sporadic in nature, occasionally it assumes epidemic proportions in places of heavy and frequent rainfall leading to mortality of young plants (Willams *et al*. 1975). The disease causes heavy plant mortality at seedling and vegetative stages, resulting in poor plant stand and lower yield (Mishra and Shukla, 1987). Characteristic symptoms of the disease are water-soaked lesions on the leaves and slightly sunken lesions on stems and petioles, girdling of the stem and foliage drying (Vishwa Dhar *et al*., 2005).

Several workers have described inoculation techniques in phytophthora blight of pigeonopea using mycelium (Kannaiyan *et al*., 1981; Nene *et al*., 1981). To mimic the disease cycle in nature, the study needs infective propogule of sporangia and zoospores to cause the infection in the host plants. However induction of zoospores and sporangia in culture plate is troublesome and also limited information are available on protocol for sporulation of sporangia and zoospores in the laboratory, hence the present study was undertaken for standardizing the laboratory protocol and standardization of inoculum density for inoculation.

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MATERIALS AND METHODS

Fungal culture

Pigeonpea plants showing the typical symptoms of Phytophthora blight were collected from the Pigeonpea fields of ICRISAT, Patancheru. The isolation of pathogen was done according to tissue segment method (Rangaswamy, 1958). Stem bits consisting of 50 per cent infected and 50 per cent healthy were surface sterilized using 1 per cent sodium hypochlorite (NaOCl) for 60 seconds and then washed in sterile water thrice. The stem bits were blot dried and plated

on petri plate containing V8 juice agar media (Himedia, Mumbai, India) amended with PARP antibiotics (pimarcin 400 µL; ampicillin 250 mg; rifampicin 1000 µL and pentachloronitrobenzine 5 mlL-1 media). Plates were incubated at 30°C in the 12 h/12 h day-night photoperiod for 5 days. Putative Phytophthora colonies were selected and confirmed by cultural and morphological characteristics as described by (Erwin and Ribeiro, 1996). Sporulation of the fungus was observed under Olympus CX41 phase contrast microscope with Q image micropublisher 5.0 RTV digital camera. The fungus was subcultured and maintained on tomato extract agar slants under *in vitro* at 15 ± 1°C in dark condition by regular sub culturing after 15–20 days. Pathogenicity of fungus was established by proving the Koch's postulates on the highly susceptible cultivar ICP 7119 (Reddy *et al*. 1991) in controlled conditions. Virulence of the pathogen was maintained by transferring the pathogen through susceptible host after every 60 days.

Protocol-Sporangia and zoospore stimulation

A thorough survey of literature was done for methods of induction of sporangia and zoospores from *Phytopthora* sp under artificial conditions. Taking clue from literature, total of six methods described for various species of Phytopthora were tried for the induction of sporangia and zoospore in the test pathogen. On experimentation, it was observed that, induction of sporangia and zoospore was not observed in any of methods. Hence, it was proposed to design a new method for induction. The detailed procedure (Fig 1) is hereunder.

Five day old culture of pathogen grown on tomato extract agar medium was used for induction of sporangia and zoospores. Mycelial disc of 6 mm diameter from the periphery was cut using a cork borer and transferred to Petri plate containing 15 ml of 20% diluted sterilized tomato extract broth. Inoculated Petri plates were incubated at 30 °C for 24 hours under alternate cycle of 12 h fluorescent light (2000 Lx) and dark period. The tomato extract broth was decanted, mycelial bits were washed with sterile distilled water for 2-3 times and 15 ml of sterile distilled water was added as replacement of tomato extract broth to the Petri plate and incubated at 30°C for 24 hours under 12 hours of fluorescent light (2000 Lx) and 12 hours of dark. Induction of sporangia was initiated after 24 hours and abundant production was observed within 36 hours primarily towards the margin of the colony and about 70-150 zoospores were released within 6-12 hours from zoosporangium. The sequence of release of zoospores from zoosporangia was enumerated in Fig 2. To determine the zoospore load, one drop of spore suspension was placed on a haemocytometer and the number of zoospores was counted in 5 squares. The number of zoospores per ml was calculated with a haemocytometer, using the formula given by Pathak (1984).

$$\text{Number of spores/ml} = (N \times 1000)/X,$$

Where,

N = Total No. of spores counted/No. of squares and

X = Volume of mounting solution between the cover glass and above the squares counted.

Isolation of mono zoospore and proving its pathogenicity

The isolation of mono zoospores was done using the method of Thakur *et al*. (1998). The fresh zoospores were harvested from water suspension and diluted (1 to 2 zoospores/field of microscope) suspension (0.5 ml) was spread uniformly over the surface of sterile water agar medium (1%) in petri plates and the excess suspension was drained off. Single, well-isolated zoospores were marked on water agar using a dummy objective under the microscope (10×). Single zoospores were picked up with the help of a flat-tipped needle and transferred onto the petri plate containing tomato extract agar and the growth was observed in petri plate. Further, pathogenicity of suspension (1×10^5/ ml) was tested using a susceptible pigeonpea variety ICP 7119 at 10 day old seedling in controlled conditions.

Zoospore concentration-seedling infection

Susceptible cultivar (ICP 7119) seeds were surface sterilized with 2% sodium hypochlorite for 2 minutes and rinsed in sterile water in order to wash off sodium hypochlorite. Surface sterilized seeds were grown in the plastic pots (5 inch) filled with a mixture of sterilized alfisol comprising of 60% sand, 33% clay and 7% silt (Kannaiyan *et al*. 1981) kept in a greenhouse maintained at 28-30 °C for 10 days. Ten day old seedlings were inoculated with zoospores with different inoculum concentrations *viz*., $1x10^{-2}$,$1x10^{-3}$, $1x10^{-4}$ and $1x10^{-5}$ zoospores/ml. Five ml of inoculum containing different concentrations of zoospores was sprayed on seedlings. Before inoculating the plants, pots were flooded with deionised water up to collar region of plants. The incubation period and disease incidence was recorded everyday up to the complete mortality.

Temperature and RH on number zoospores

An experiment was conducted to determine the effect of various ranges of temperatures (20, 25, 30, 35, 40°C) and relative humidity (50, 75, 85, 95, 100%) using protocol of diluted tomato extract broth. The number of zoospores at different treatment was calculated with a haemocytometer using the formula given by Pathak (1984).

RESULTS AND DISCUSSION

Stimulation of sporangia and zoospore

Production of sporangia and zoospores is the principle means by which the numbers of infective units are increased and accounts for the rapid regeneration time of *Phytophthora* species. Sporangia together with zoospores are the principal means of dispersal of the *Phytophthora* spp. and as the most potent means ensuring penetration of the host (Weste and Vithanage, 1979). In the present study, various methods given by earlier workers were attempted for induction of sporangia and zoospores of *P. cajani* and results (Table 1)

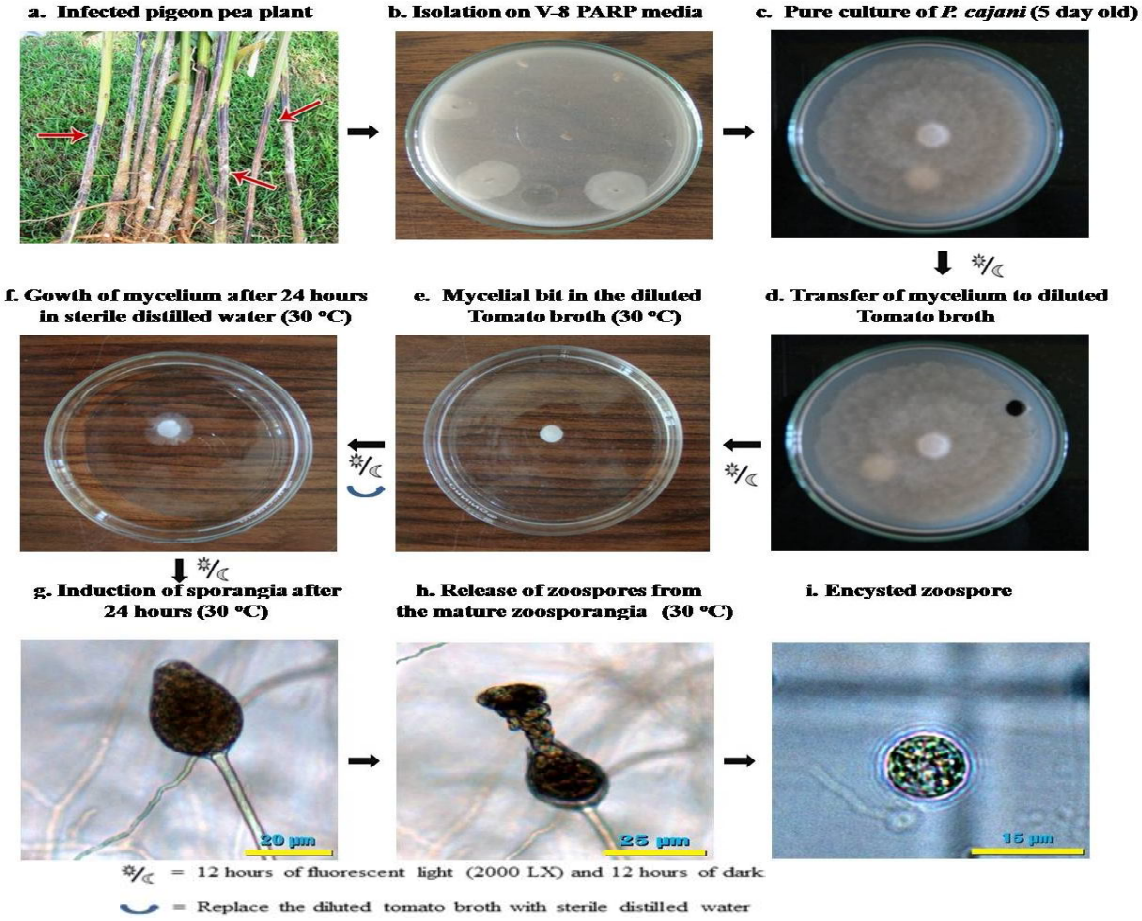

Fig 1: Protocol for induction of sporangia and zoospores in *Phytophthora cajani.*

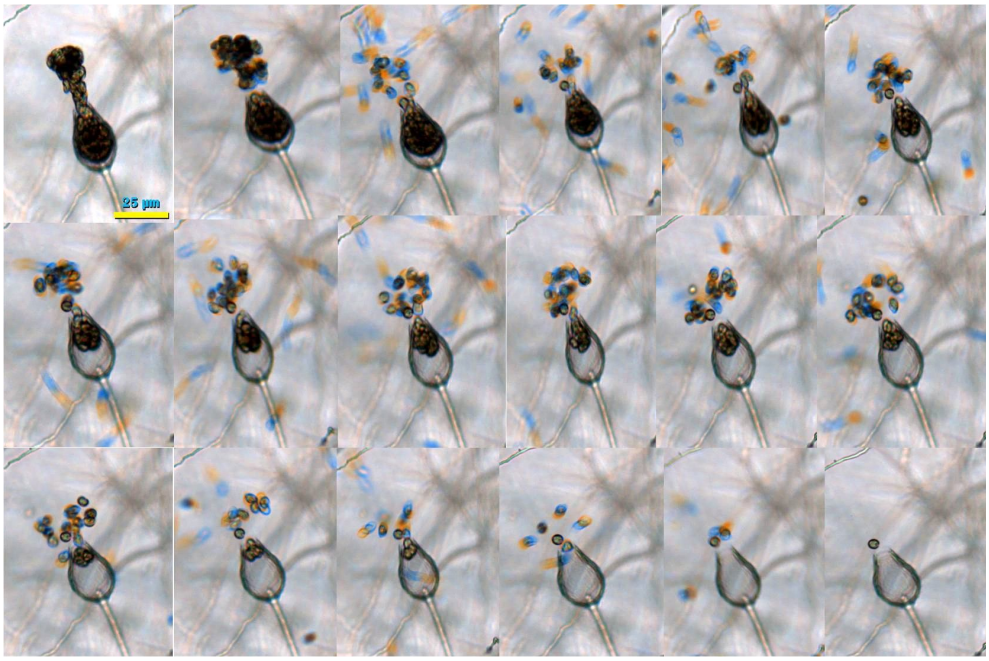

Fig 2: Sequence of zoospore release from zoosporangium of *Phytophthora cajani.*

Note: In frame 1: Emitted zoospores are retained in the membranous sac. In frame 2: the membranous sac has broken and zoospores are released. Bar = 25µm.

Table 1: Protocols attempted for induction of sporangia and zoospores of *Phytophthora cajani*.

Method	Asexual spores*		Male and female gametes*		Sexual spore*	Resting spore* Chlamydo spore
	Sporangia	Zoospore	Antheridium	Oogonium	Oospore	
KNO_3 solution (Schiffman and Cohen, 1968)	-	-	-	-	-	-
Sun flower, Pea, Corn and Soybean seed media (Felix, 1962)	-	-	-	-	-	-
Frozen pea medium (Dance, 1975)	-	-	-	-	-	-
Flooding on cultures (Ribeiro and Baumer, 1977)	-	-	-	-	-	-
V-8 broth (Kannaiyan *et al.* 1992)	-	-	-	-	-	-
Rape seed extract agar (Satour and Butler, 1968)	-	-	-	-	-	-
Diluted tomato extract broth (Present study)	+	+	+	+	+	-

*Mean of five replications, '-' = absent, '+' = present.

reveled that, none of the protocols are able to stimulate the sporangia and zoospores. Hence, the alternative method for induction of spornagia and zoospore was attempted. Taking clue from the study of culture media and mycelial growth of *P. cajani,* tomato extract agar produced maximum growth therefore; further probed to develop a protocol for maximum production of sporangia and zoospores by diluting tomato extract broth with fresh water. It was found that diluted tomato extract broth could induce abundant sporangia and zoospores within 24 hours of incubation. Further same methodology could also induce the male and female gametangia *viz.,* antheridium and oogonium and sexual spore oospore within 48 hours. The induction of sporulation of *P. cajani* using diluted tomato extract broth *in vitro* is first of its kind. The induction of sporulation in tomato broth could be due to its precise availability of nutritional requirement for *Phytophthora* in the medium as reported earlier by Grant *et al*., 1984.

On observation found that the most important factor involved in the process of sporulation by *P. cajani* is sudden reduction in food supply which forced the fungus to sporulation stage instead of the vegetative stage and stimulation of sporangial production thus initiating the cycle of spore formation. Major advantages of this protocol are easy handling, reproducible and rapid for obtaining the zoospores within 36 hours and enable us the inoculating large numbers of pigeon pea genotypes for resistance breeding against the Phytophthora blight disease. Any information relating to this zoospore is significant to understanding the pathogen and host x pathogen x environment interaction and ultimately to manage the disease.

Diplanetism

Diplanetism, the phenomenon observed in some members of the *Oomycetes*, of there being two distinct motile phases, with morphologically different zoospores formed in each. In the study *P. cajani* showed a diplanetism mechanism, in that one zoospore may germinate directly forming an emerging tube and producing secondary zoospores (Fig 3). The report of *P. cajani* producing diplanetism mechanism is first of its kind. Similar report in *Phytophthora ramorum* doneby Moralejo and Descals (2011), where *P. ramorum* displays monomorphic diplanetism and microcyclic sporulation, in that cysts may germinate directly forming a emerging tube or indirectly by releasing secondary zoospores. These Secondary zoospores do not differ morphologically and can infect hosts. However, symptom expression is delayed and infectivity is lower than for primary zoospores. The diplanetism mechanism in the pathogen may provide a second opportunity for host infection and may increase the chance of dispersal of pathogen in the soil (Moralejo and Descals, 2011).

Temperature and RH on number of zoospores using diluted tomato extract broth

Phytophthora has a complex asexual life cycle with distinct multiple infectious propagules which include the mycelium, sporangia and zoospore cysts (de Souza *et al*. 2003). Each of these asexual developmental stages is crucial for plant infection and disease development (Hardham, 2001). Different environmental factors such as temperature, water relations, physical and chemical conditions and interacting combinations of these factors have been reported to influence *Phytophthora* pathogenesis (Duniway, 1983). Moreover, the geographic distribution and seasonality of Phytophthora diseases are thought to depend on the constraints of any of these environmental factors on any or all of the life cycle stages (Duniway, 1983). Of the environmental factors, temperature has been reported to have greatest influence on growth, reproduction and pathogenesis of *Phytophthora* spp. (Matheron and Matejka, 1992; Timmer *et al*. 2000).

Studies on the influence of temperature and RH on sporangial production by *Phytophthora* spp. is an important prerequisite for developing disease predictive models in the field. Hence, an attempt made to study the impact of temperature (20, 25, 30, 35 and 40°C) and RH (50, 75, 85, 95 and 100 %) on zoospore production using the diluted tomato extract broth. The results (Fig 4A) revealed that the greatest number being produced at 30°C (183×10^3 per ml), whereas few zoospores were produced at variation of 30°C *viz*., 35°C (86×10^3 per ml) and 20°C (112×10^3 per ml), whereas at 40°C production of zoospore was nil. The results implied that the ambient temperature of 30°C has a more critical effect on reproduction of pathogen. Similar reports were made in other species of Phytophtohra *viz*., *P. citrophthora* and *P. parasitica* (Matheron and Matejka, 1992);

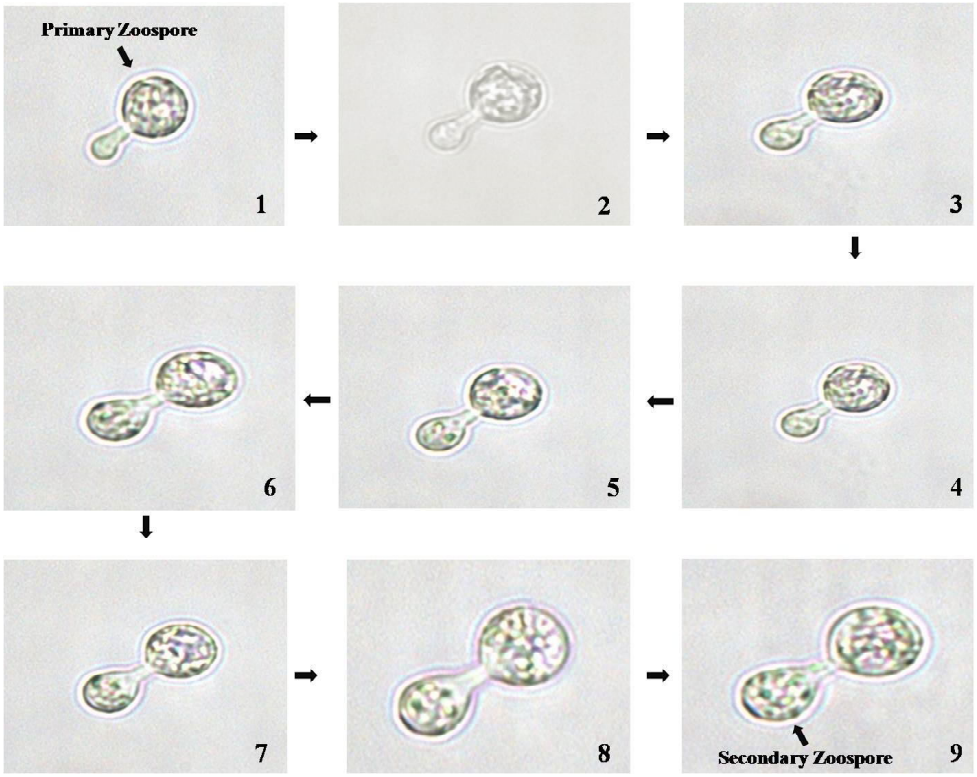

Fig 3: Diplanetism mechanism of *Phytophthora cajani.*

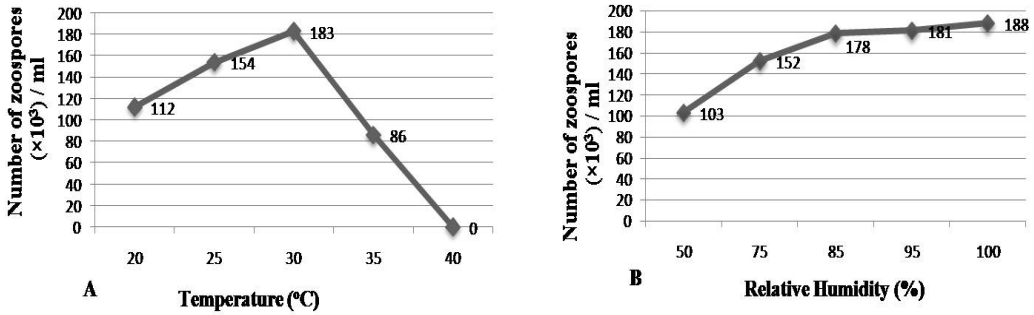

Fig 4: Relationship between temperature and RH on induction of zoospores.

P. infestans (Mizubuti and Fry, 1998) and *P. palmivora* (Timmer *et al*. 2000).

Among all the RH levels, RH of 100 % induced highest number of zoospores (188 × 10^3 per ml) and statistically superior over others (Fig 4B). Similar observations has been reported in *Phytophthora pseudosyringae* sp. nov (Jung *et al*. 2003), *P. infestance* (Minogue and Fry, 1981) and *P. capsici* (Granke and Hausbeck, 2010b) where RH played great role in influencing the amount of zoospore formation.

Zoospore concentration and plant infection

The optimum quantity of inoculum should be selected to characterize better relationship between inoculum concentration and infection (Fraedrich *et al*., 1989; McIntyre and Taylor, 1976; Milholland *et al*., 1994). Quantity of inoculum is bound to influence the disease incidence and infection takes place only when minimum inoculum potential of the pathogen is present in the soil.

Although, information is available on the numbers of zoospores required for infection of plants or plant parts on different species on *Phytophthora* (Halsall, 1977; Hickman, 1970; Pratt *et al*., 1975), the information on the quantities of zoospores required to infect pigeonpea plants under flooded conditions is very limited. The study established the quantitative the relationship between numbers of zoospores of *P. cajani* and infection in pigeonpea. The study indicated that, with the increase in concentration of zoospores there was substantial increase in the infection of plant. Of all concentrations, $1x10^{-5}$ zoospores/ml induces maximum infection of plant (100 %) and shortest incubation period (24 hours), whereas only 6.6, 19.6 and 69.4 percent infection was observed at $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-4}$respectively (Fig 5). Further, incubation period is delayed as reduction in inoculum load. Shortest incubation period at higher concentration of zoospores caused due to initial infection by large numbers of zoospores and delayed incubation period is because of

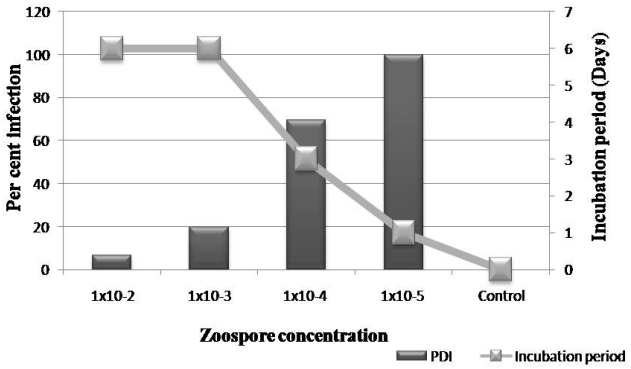

Fig 5: Zoospore concentration and plant infection.

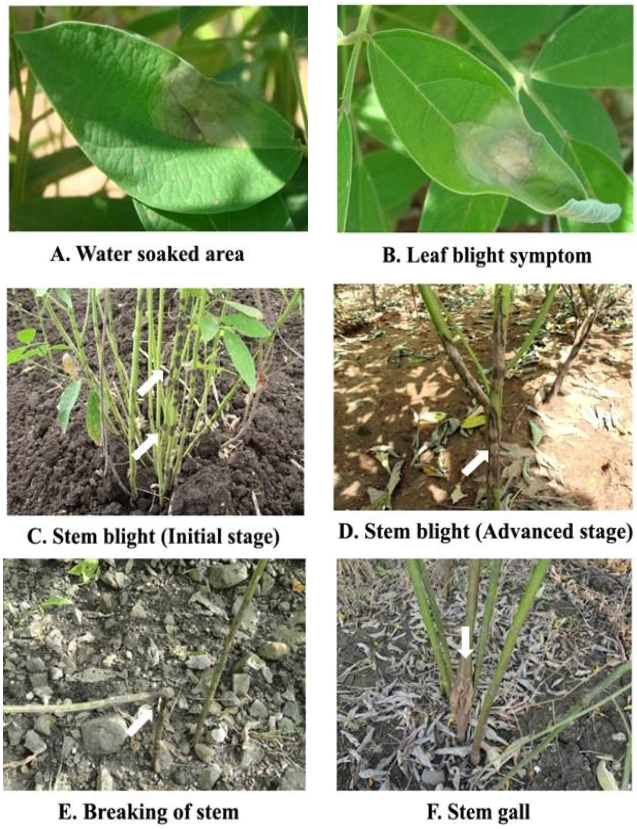

Fig 6: Symptomology of Phytophthora blight disease of Pigeon pea.

initial infection by low levels of zoospores. The results of this study are in general agreement with work with *P. parasitica* var. *nicotianae* on tobacco (Gooding and Lucas, 1959), *P. palmivora* on papaya (Ko and Chan, 1974). The amount of initial inoculum determines the amount of disease. The disease increased with the increase in an amount of inoculum concentration as availability of infection courts and at certain stage addition of extra inoculum did not increase the amount of disease. This could be due to saturation of all the infection courts Van der Plank (1975).

Symptomology of disease

During the periodical survey at research farm, ICRISAT the symptoms were recorded and presented in Fig 6. Infected young seedlings showed crown rot symptoms soon after their emergence, later collapsed and died within 4 days. In older plants, *i.e.* on 1 to 2 month old plants, water soaked lesions of varied size and shape appeared on the primary and trifoliate leaves (Fig 6a). As the disease progressed, the entire foliage blighted (Fig. 6b). Brown, dark brown or black lesions appeared on the collar region above the ground level on the main stem and branches (Fig 6c; 6d). During severe infection, the lesions increased in size and encircled the stems causing them to shrink. The stem above the lesion girdled dried out and broke at the infection site (Fig 6e). In late infections, lesions on stems developed into cankers or galls and the infected bark cracked. (Fig 6f). Williams *et al*. (1975); Nene *et al*. (1979); Vishwa Dhar *et al*. (2005) and Pande *et al*. (2011) observed similar type of symptoms in the field and under greenhouse conditions.

CONCLUSION

The study established new development in relation to methodology for production of zoospores which enable the inoculating large numbers of pigeonpea genotypes for resistance breeding. Information relating to biology of pathogen is significant in managing the disease effectively.

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